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(54) Title: METHOD FOR PREVENTION OF XENOGRAFT REJECTION BY TRANSPLANT RECIPIENTS

(57) Abstract

Hyperacute rejection (HAR) and accelerated acute rejection (AAR) of discordant cardiac xenografts is prevented by inhibiting complement by treatment of the donor and the xenograft to be transplanted with soluble form of the human complement receptor (sCR1). An exemplary model utilizing a pig heart transplanted into a monkey is provided.

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METHOD FOR PREVENTION OF XENOGRAFT REJECTION BY TRANSPLANT RECIPIENTS

BACKGROUND OF THE INVENTION

1. Field of the Invention

The present invention relates generally to the field of transplantation and rejection and specifically to a method for preventing xenograft rejection by transplant recipients by inhibition of complement.

2. Background of the Invention

Although the immune response is often perceived as beneficial, in certain circumstances the immune response to an antigen can actually be harmful to the animal in which the immune response occurs. Examples of situations where the immune response creates conditions where the animal is subject to serious pathological sequelae are in areas such as graft versus host (GVH)[1] rejection and host versus graft (HVG) rejection, certain autoimmune diseases, such as lupus erythematosus, insulin-dependent diabetes mellitus, multiple sclerosis, myesthesia gravis, and rheumatoid arthritis.

The utilization of organs from nonhuman donors is an appealing solution to the increasing shortage of organs available for clinical transplantation. Although xeno-transplantation from primate donors has been performed with limited clinical success, the use of distantly related species, such as members of the porcine family, avoids ethical dilemmas, potential virus transmission, and limited availability associated with the use of primates as xenograft (Xg) donors. However, xenotransplantation using distantly related species is currently prohibited by the occurrence of hyperacute rejection (HAR), a process that leads to irreversible Xg damage and loss within minutes to hours. HAR is thought to be mediated by the binding of naturally occurring xenoreactive antibodies to the endothelium of the Xg, in particular, donor vascular endothelial cells, with subsequent activation of the classical pathway of complement (C). It has been shown that a predominate specificity of these antibodies is to the

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Abbreviations used herein are: **AAR**, accelerated acute rejection; **Ab**, antibody; **AP50**, alternative pathway f complement activation; **C**, complement; **CCS**, cyclosporin, cyclophosphamide, and steroids; **CH50**, total hemolytic activity of complement; **cyn** , cynomolg us monkey; **HAR**, hyperacute rejection; **IVC**, inferi r vena cava; **POD**, postoperative day; **sCR1**, soluble complement receptor type 1; **WBC**, white blood count; **Xg**, xenograft.

oligosaccharid moiety galactose (α1-3) galactose for primate recipients. Alternative C pathway activation also contributes to HAR in some species combinations. The complement cascade is activated following the binding of xenoreactive antibodies to donor tissue. This leads to endothelial activation, thrombosis, intravascular coagulation, edema, and eventually loss of function of the transplanted xenograft. However, if xenoreactive natural antibodies are eliminated, the presence of complements is still adequate to mediate a rejection event presumably via the alternative pathway.

The central role of C activation in the HAR process has been suggested by the observation of prolonged Xg survival in recipients depleted of C by treatment with cobra venom factor (CVF). However, the clinical use of CVG is likely to be associated with unacceptable morbidity. In addition, the humoral immune response elicited against the foreign CVF protein might limit its effectiveness when repeated.

A number of approaches to limiting HAR by inhibiting complement have been considered. Expressing human cell surface complement regulatory proteins such as MCP, DAR or CD59 in the vessels of transgenic pigs is now being actively pursued. This approach has the advantage of localizing complement inhibition to the affected sites of HAR, namely the endothelium of the graft. Alternatively, a simple treatment consists of administering (intravenously or intraperitoneally) complement inhibitory therapeutics to the recipient of the xenograft.

One such agent is soluble complement receptor type 1 (sCR1), a potent inhibitor of both the classical and alternative pathways of complement. sCR1 is a truncated recombinant form of the naturally occurring human protein CR1 (CD35, C3b/C4b receptor) that was constructed and expressed. sCR1 binds to C3b and C4b, thus inhibiting the C3 and C5 convertases. Thus, sCR1 prevents the release of the pro-inflammatory anaphylatoxins C3s and C5s and prevents the downstream assembly of the membrane attack complex (MAC, C5b-9) when initiated by either pathway of activation of the complement system. By acting as a co-factor for Factor I, sCR1 promotes the degradation of C4b and C3b and is thus released to recycle in the inhibitory process. These properties make sCR1 an ideal candidate to counteract the consequences of complement activation leading to xenograft rejection.

Pruitt, *t al.*, (*Transplantation*, 57:363, 1994) d monstrated, in an *in vivo* pig-to-primat heterotopic cardiac xenotransplantation model, that a single intrav nous

bolus of sCR1 administered to th recipient immediately before Xg reperfusion markedly inhibited total and alternative pathway serum C activity and prolonged Xg survival to between 48 and 90 hours. Untreated controls underwent HAR within 1 hour or less. Further studies demonstrated that continuous infusion of sCR1 after xenograft transplantation with no other treatment resulted in further prolongation of xenograft function, but ultimately rejection occurred after 5-7 days despite reduced C activity.

Previous attempts to overcome the obstacles of xenograft transplants between widely disparate species (discordant xenotransplantation) have focused on methods that deplete or inactivate complement and/or antibody. Procedures to date have not been applicable clinically because of the unacceptable risk of extensive immunosuppression, which most often results in serious or lethal infection. Alternative approaches have involved the development of "transgenic" pigs, which express human regulators of complement that would locally inhibit complement activation.

Once complement mediated HAR has been inhibited, the full spectrum of cellular and antibody-mediated inflammatory and immune responses characteristic of acute and chronic rejection will need to be canceled. But the fact remains that if xenotransplantation is to become a clinical reality, a clinically relevant means of inhibiting complement activation will be required. sCR1 provides such a therapeutic option and an option where the dosing regimen is under the control of the physician and can be adjusted in response to the need of the patient.

SUMMARY OF THE INVENTION

The present invention provides a method for providing a clinically acceptable means of replacing human organs by transplantation of non-human xenografts. The invention provides donor xenografts treated with therapeutic levels of sCR1 prior to and following recipient xenograft transplantation.

In a preferred embodiment, the invention provides a method of xenograft preparation or transplantation comprising treating a xenograft with a complement-inhibiting amount of sCR1 prior to transplantation of th x nograft; and transplanting the xenograft to a recipient treated with a combination of immunosuppressive agents and sCR1.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention involves the application of a recombinant human protein termed "soluble complement receptor type 1" (sCR1) for use in a method of pretreating a donor and/or the donor xenograft before transplantation to prevent early rejection reactions due to complement activation resulting from antibody binding and/or complement dysregulation. Prior to transplantation, the donor or the specific donor xenograft is treated with sCR1 to attain therapeutic levels by the time of donor xenograft removal and transplantation. In addition, upon removal and prior to transplantation, the donor xenograft is treated again with SCR1.

The invention also presumes treatment of the recipient prior and subsequent to transplantation with an appropriate combination of genetically altered donor xenografts and/or immunosuppressive agents that will inhibit complement activation as well as the host antibody and cellular immune responses to the donor xenograft.

An example of the way in which the invention may be applied to pig heart donors for human recipients is as follows: beginning one week prior to the transplantation, the recipient may be treated with cyclophosphamide to reduce the potential for evoked antibody responses. An immunosuppressive dose of cyclosporine or FK506 may be started shortly (1-3 days) before transplantation to enhance graft acceptance. Immediately prior to xenograft placement, the donor is treated with sCR1 to attain therapeutic levels by the time of donor xenograft placement. Immediately prior to transplantation, the donor xenograft is flushed with a solution containing sCR1. Following transplantation by standard surgical techniques, the patient is maintained on routine immunosuppression using cyclosporine or FK506, cyclophosphamide, and steroids plus sCR1. Based on clinical signs and symptoms related to immune responsiveness, various of the immunosuppressants are reduced in dosage. In a preferred embodiment, cyclosporine A is administered to the recipient prior to and following xenotransplant. Steroids, such as depomedrol are administered following xenotransplant and cyclophosphamide is administered both prior to and following transplantation.

The immunosuppressive agent used according to th method of th inv ntion is an agent such as Cycl sporine A (CsA), however other agents which cause immune suppr ssion, such as rapamycin, desoxyspergualine, and FK506 or functional equival nts of thes compounds, may also be utilized. CsA is preferably

administered by injection at an immunosuppressive d se. The duration of CsA treatment may be in the range from about 2 to about 20 days.

The sCR1 or immunosuppressive agent is administered by any suitable means, including parenteral, subcutaneous, intrapulmonary, and intranasal administration, and if desired for local immunosuppressive treatment, intralesional administration (including perfusing or otherwise contacting the graft with the sCR1 or immunosuppressive agent prior to transplantation). Parenteral infusions include intramuscular, intravenous, intraarterial, or intraperitoneal administration. In addition, the immunosuppressive agent is suitably administered by pulse infusion, particularly with declining doses of the immunosuppressive agent. Preferably, the dosing is given by injections, most preferably intravenous or subcutaneous injections, depending in part on whether the administration is brief or chronic.

Although the example of the invention illustrates transplantation of a pig heart into a monkey, it is understood that any xenograft can be transplanted. For example, other transplantable xenografts include cornea and kidney. Further, while the pig is a preferred donor, other donors may also be used including organs or tissues grown or maintained in *in vitro* culture. The human is the preferred recipient.

As used herein, "substantially contemporaneously" refers to the time at which the immunosuppressant is administered to the recipient in relation to the time at which the xenograft is transplanted. For example, a heart transplant recipient may receive CsA for a short time prior to and immediately following the transplant for about 10-16 days, preferably about 14 days. In general, where transplant grafts are involved, the immunosuppressive agent can be administered from about 1 day to about 90 days before the transplant and until about 7 days to about 90 days after the transplant. Preferably, the immunosuppressive agent is administered from about 7 days to about 28 days before until about 7 days to about 28 days after.

To determine the amount of sCR1 to administer to the donor, *e.g.*, pig, and the amount of sCR1 to treat the xenograft with, complement activity in the donor is first measured. A "pre-assay", pre-existing, complement activity is determined by CH_{50}, a standard procedure known to those of skill in the art (see for Example, Manual Clinical Immunology, current edition). The donor pig, for example, is then treated with sCR1 to reduce complement activity t about 10% of the normal complement activity level in that donor. B cause the activity in each donor varies, individual donors should

be assayed independently. Therefore, as used herein, the term "complement-inhibiting" amount refers to that amount of sCR1 that inhibits complement activity in the range of about 90%.

It is recognized that potential donors can be screened and selected depending on whether or not the donor is an inbred or outbred strain for example. An outbred strain would preferably be measured on an individual basis, while an inbred strain could be measured for complement activity on a strain basis. All assays still rely on the CH_{50} as a standard measurement for complement activity. The term "donor" refers to an animal or culture from whom a xenograft is taken; while the term "recipient" refers to an animal or cultural in whom the xenograft is placed. The term "placement" refers to the surgical transplantation of an organ or tissue *in vivo* or *in vitro*. In a preferred embodiment, the "donor" is a pig or member of the porcine family and the "recipient" is a human. The term "pig" or "porcine" refers to a wild or domestic mammal of the superfamily Suoidea in the order Artiodactyla. The term "animal" refers to an organism that reproduces. The term "culture" refers to *in vitro* maintenance of a cell, tissue, organ, or organism.

The method of the invention includes treatment of the recipient with an "antibody-inhibiting" amount of cyclophosphamide, or its equivalent to reduce the potential for evoked antibody responses due to donor antibodies. "Therapeutically effective" as used herein, refers to an amount of a composition that is of sufficient quantity to ameliorate the state of the recipient so treated. "Ameliorate" refers to a lessening of the detrimental effect of the disease state or disorder in the patient receiving a composition. Although the subject of the invention is preferably a human, it can be envisioned that any animal can be treated using the method of the present invention. The term "modulate" means enhance, inhibit, alter, or modify the disease state or disorder in the patient.

A therapeutic approach included within the invention involves administration of sCR1 recombinant protein produced by any conventional recombinant protein administration technique, to the site of the xenograft, at the site where C cells may accumulate (for example, by injection), or administered systemically. The sCR1 recombinant protein may also be targeted to specific cells or receptors by any methods of gene delivery and gene expression known to those in the art. The actual dosage of sCR1 recombinant protein depends on a number of factors, including the size and health of the recipient of the xenograft, but, generally, sufficient sCR1 to reduce C activity

to less than 10% of pre-operativ l vels as measured by daily CH_{50} and AP_{50} assays, are administered per day to an adult in any pharmaceutically-acceptable carrier.

"Gene delivery" means transportation or transfer of a composition or formulation inside of or into contact with a target cell so that the composition or formulation is capable of being taken up by means of a cytotic process (*i.e.*, pinocytosis, endocytosis, phagocytosis, macrocytosis etc.) into the interior or cytoplasmic side of the outermost cell membrane of the target cell where it can subsequently be transported into the nucleus of the cell in such functional condition that it is capable of achieving detectable gene expression for a period of time and in such an amount to produce a detectable biologically beneficial effect.

"Gene expression" means the process, after delivery into a target cell, by which a nucleotide sequence undergoes successful transcription and translation such that detectable levels of the delivered nucleotide sequence are expressed in an amount and over a time period so that a functional biological effect is achieved. As used herein, gene expression can refer to, but is not restricted by (either explicitly or implicitly) the following examples. A sCR1 nucleic acid sequence is delivered and expressed in targeted cells such that the targeted cells increase, decrease, or are inhibited in the production of sCR1 protein or sCR1 RNA, thus: either enhancing phagocytosis, inhibiting phagocytosis, enhancing the complement portion of the immune system or dampening the complement portion of the immune system and subsequently leading to a beneficially detectable biological effect or outcome.

The following examples are intended to illustrate, but not limit the invention. While they are typical of those that might be used, other procedures known to those skilled in the art may alternatively be used.

EXAMPLE 1

THE RELATIVE CONTRIBUTION OF C AND PREFORMED XENOREACTIVE NATURAL ANTIBODY (NAB) TO HAR OF DISCORDANT GUINEA PIG (GP) HEART XENOGRAFTS

Complement (C) activation via classical and alternative pathways can play a key role in hyperacute rejection (HAR) of discordant cardiac xenografts. We have used two approaches to study this problem; inhibiting C at the level of terminal component activation, and inhibiting multiple components involved in both pathways. These approaches have permitted a better understanding of the role of several specific C components in mediating HAR and the effects of their inhibition alone and in combination with other immunosuppressive agents in several different models of cardiac xenotransplantation.

Using multiple rat strains to evaluate the relative contribution of C and preformed xenoreactive natural antibody (NAb) to HAR of discordant guinea pig (gp) heart xenografts, we demonstrated that a congenic strain of PVG rats with a profound deficiency in C6 (PVG/C-) resulted in the lack of membrane attack complex (MAC) formation. This specific deficiency is associated with the complete absence of HAR, despite high titers of NAb: gp heterotopic heart xenografts were rejected by congenic PVG/C+ recipients within 0.5 ± 0.2 hr compared to 45± 9 hr by PVG/C- recipients.

The pattern of rejection seen in C- recipients was one of accelerated acute rejection, with abundant neutrophils and platelets. Attempts to further prolong survival in C6 deficient recipients by inhibition of neutrophil adhesion were partially successful; the use of antibody to CD18β, a component of Mac-1, LFA-1, and gp150,95, showed no benefit, whereas treatment with NPC 15669, a leumedin that blocks MAC-1 upregulation, showed significant prolongation beyond controls.

The degree and source of C6 activity required for HAR was examined by transplanting C+ recipients with C- livers, yielding full reconstitution of C6, and reconstituting PVG/C6- rats with bone marrow from C+ donors, yielding 10% reconstitution of C6.

In the former case, extrah patic sources of C6 were sufficient to yield HAR, whereas in the latter, HAR was delayed to 9±3 hr. S lectiv d pletion of C6 by anti-C6

antibody treatment has reproduced the effect seen in C6 deficient recipients. LEW rats were treated with rat-anti-rat C6 antiserum to deplete C6 prior to gp cardiac xenotransplantation, and afterwards to inhibit natural reconstitution. Treated rats showed abrogation of HAR (38±1 hr) compared to normal serum treated controls (1±0.7 hr). Use of the purified IgG fraction of the anti-C6 serum likewise prevented HAR. The fact that C6 deficiency can inhibit acute (and hyperacute) allograft rejection, raises the possibility that this approach may be a useful adjunct in promoting long-term xenograft survival.

EXAMPLE 2

SINGLE BOLUS TREATMENT WITH sCR1 IN RODENT AND PRIMATES

An approach to overcome HAR has been to inhibit both the classical and alternative pathways at steps earlier than C6 using the recombinant soluble form of human C receptor 1 (sCR1), which has both decay accelerating and co-factor activity. Initial work in the gp to rat cardiac xenograft model as described in example 1, demonstrated a dose dependent association between sCR1 treatment and inhibition of HAR. Subsequently, using an ex-vivo model of pig heart perfusion with human blood, we found that single dose treatment with sCR1 abrogated C activation and prevented HAR.

Likewise, a single bolus treatment with sCR1 of cynomolgus monkeys at the time of heterotopic pig heart transplantation, with no other treatment or preparation, resulted in the abrogation of hyperacute rejection compared to vehicle treated controls: 70±18 vs 0.8±0.2 hr. At the time of rejection, sCR1 treated recipients showed reconstitution of classical and alternative C pathway activity and elevation of NAb, especially IgG (Pruitt, *et al. Transplantation* 57:363, 1994).

A single 15 mg/kg IV bolus of sCR1 given immediately prior to Xg reperfusion prolonged porcine cardiac Xg survival to 48-90 hr (vs 51 hr for controls). C activity slowly returned after the single bolus and C deposition was noted in the Xgs at rejection in these sCR1 treated recipients.

EXAMPLE 3

CONTINUOUS TREATMENT WITH sCR1 IN PRIMATES TO PREVENT HAR

The current example demonstrates the effect of sustained C inhibition using sCR1 on HAR in primates.

Two cynomolgus monkeys underwent heterotopic porcine cardiac xenotransplantation. Prior to Xg reperfusion, each received an initial 25 mg/kg bolus of sCR1, followed by a continuous 40 mg/kg/day infusion of sCR1. This treatment regimen resulted in sustained C inhibition (<20% preoperative levels) as assessed by daily CH50 measurements.

In the first recipient, porcine Xg survival was 5 days (120+ hours). On day 3, the infusion catheter blocked, depriving the recipient of sCR1 for approximately 8 hours. At that time, an additional bolus of sCR1 was administered, and the infusion was restarted. In the second recipient, who experienced no interruption of sCR1 infusion, Xg survival was 7 days (168+ hours).

Continuous infusion of sCR1 with no other treatment resulted in further prolongation of xenograft function, but ultimately permitted rejection at 5-7 days despite reduced C activity. At the time of rejection, the graft showed extensive IgM and IgG deposition, with abundant neutrophils and macrophages but modest T cell infiltration. NAb levels of both IgG and IgM were both elevated at rejection. Biopsies of the functioning cardiac Xgs were obtained at various time points. At the time of rejection, Xgs were remarkable for a cellular infiltrate composed predominantly of neutrophils (myeloperoxidase+) and macrophages (MAC387+, KP1+). Some infiltration by these cell types was noted as early as the day 2-3 biopsies. Infiltration by CD3+/CD4-cells was also noted.

These data confirm the important role of C activation in HAR of porcine cardiac Xgs by primates and indicate the usefulness of sCR1 as prophylactic agent for prevention of xenotransplantation rejection. These data also suggest that once C-mediated HAR has been inhibited, infiltration of the Xg by neutrophils and macrophages may be the next barrier to successful longer-term Xg survival.

EXAMPLE 4

AAR PREVENTION USING SCR1 AND TRIPLE THERAPY WITH STANDARD IMMUNOSUPPRESSIVE AGENTS

In the previous examples, we have shown that continuous inhibition of C with sCR1 prevents HAR and precludes C activation and deposition in the xenograft (Xg). Nevertheless, xenografts were rejected within 1 week post-transplantation in association with a rise in serum xenoreactive Ab and a cellular infiltrate composed primarily of neutrophils and macrophages. Thus, although HAR can be prevented by C inhibition, C independent processes characterized as AAR ultimately result in graft destruction. The present example elucidates the immune events that occur in a pig-to-primate cardiac Xg model when continuous C inhibition and CCS was employed.

To determine the significance of these immunologic changes in the ultimate rejection of the Xg we have attempted to prevent their occurrence by combining triple therapy using standard immunosuppressive agents (CCS; cyclosporin, cyclophosphamide, steroids) with a regimen of continuous sCR1 therapy in a pig-to-primate cardiac Xg model. Our findings suggest that combined inhibition of C, xenoreactive Ab responses, and cellular immunity may be a useful approach in overcoming immune barriers to xenotransplantation.

Materials and Methods

Animals. Seven to fourteen day old piglets weighing approximately 5 kg were used as cardiac Xg donors. Adult male cynomolgus monkeys weighing 5-7 kg served as recipients. All animals were cared for in accordance to NIH guidelines under the supervision of qualified veterinarians.

Pig-to-primate cardiac Xg model. Piglets were sedated with ketamine (10 mg/kg IM), intubated, and anesthetized with inhalational isofluorane and oxygen. Using sterile conditions, a median sternotomy was performed. After ligation of the superior vena cava and inferior vena cava (IVC) the aorta was cross clamped. Cold (4_C) crystalloid cardioplegia (Plegisol; Abbott Laboratories, Chicago, IL) was injected into the aortic root until v ntricular contractions ceased. Meanwhile, th IVC was incised to vent the heart. Cold salin slush was applied t pically. After th aorta and pulmonary artery and v ins were divid d the heart was rem ved and placed in salin slush. Th pulmonary veins w re oversewn with a running 6-0 Prolene sutur (Ethicon, Somerville, NJ).

Monkeys were sedated with ketamine hydrochloride (10 mg/kg IM) and anesthetized with inhalational isofluorane, nitrous oxide, and oxygen. Under sterile technique a double lumen 7.0 Fr Hickman catheter was placed in the right internal jugular vein by cutdown and direct visualization. The catheter was then tunneled subcutaneously to exit between the scapulae and sutured in place. Postoperatively, one port of the catheter was used for continuous sCR1 infusion and the other port for blood sampling and intermittent drug administration.

The abdomen was then sterilely prepped and draped, a midline incision made, and the infrarenal IVC and aorta isolated. The aorta and pulmonary artery of the donor were anastomosed end-to-side to the abdominal aorta and IVC of the recipient, respectively, using a running 6-0 Prolene suture. Vascular clamps were removed and Xgs defibrillated with 5-10 J as necessary. Grafts were observed for 45 minutes (or until rejection) and then the abdomen was closed in layers. The recipient was kept under reverse respiratory isolation and monitored continuously. Daily brief sedation with ketamine allowed for assessment of wound integrity and graft function. Rejection was defined as complete cessation of ventricular contractions. Echocardiographic evaluation of graft function was possible in one experimental monkey.

Immunosuppressive Regimen. For all animals 25 mk/kg sCR1 (BRL55730, kindly provided by T Cell Sciences, Needham, MA) was injected into the aortic root of the donor heart after cardioplegic arrest was achieved. Recipients which were treated with sCR1 were administered 25 mg/kg IV at the start of surgery prior to placement of the vascular cross clamps and then another 25 mg/kg IV prior to graft reperfusion. This was followed by a continuous infusion at 40 mg/kg/day IV. Control monkeys received saline injections. Because of potential infection of the indwelling central line, all monkeys were treated prophylactically with Ancef (cefazolin, Bristol-Meyers Squibb, Princeton, NJ) 45 mg/kg/day IV divided every 8 hours.

CCS therapy consisted of 2 different three drug regimens designated CCS* and CCS. Both employed the same cyclosporin and steroid dosing but different cyclophosphamide dosing. Cyclosporin was given daily beginning two days prior to surgery at 10-15 mg/kg IM in order to achieve plasma levels of 400-600 ng/ml. Solumedrol 125 mg IV was given on the day of surgery and postoperative day (POD) 1. This steroid dose was then decreased to Depomedrol 1 mg/kg IM daily. CCS* consisted of daily cyclophosphamide 1 mg/kg IV beginning the day of transplantation. This dosing was inadequate to lower either the white blood count (WBC) or the

natural Abl v ls. CCS, which prevented AAR, employed both a pre perative regimen of cyclophosphamide as well as a higher daily dosage. Cyclophosphamide 10-20 mg/kg po was given every other day one week prior to transplantation. After surgery, a daily dose of 2.5-20 mg/kg IV was given to attain a WBC < 4,000 cells/mm3.

Cynomolgus monkey (cyno) 1 was a control which received CCS but no sCR1 (although the donor heart was pretreated with sCR1). Cyno 2 received sCR1 + CCS*. Cynos 3 and 4 received sCR1 + CCS.

Immunohistology. Formalin-fixed tissues were embedded in paraffin, sectioned, stained with hematoxylin and eosin, and examined by light microscopy. Tissues were also embedded in gelatin and snap frozen in a liquid nitrogen-immersed isopentane bath, and 5mm thick sections were mounted on gelatin-coated slides. After acetone fixation, sections were stained with either FITC-conjugated goat anti-human IgM, goat anti-human IgG (Sigma Chemical Co., St. Louis, MO) or goat anti-human C3 (Nordic Immunologicals, Capistrano Beach, CA). To evaluate cellular infiltrates, immunoperoxidase staining of fixed tissue sections was performed using the monoclonal antihuman antibodies KP1 (macrophages), CD3 (T cells) and myeloperoxidase (neutrophils) (Dako, Carpinteria, CA). Immunofluorescent slides were examined using a Zeiss D-7982 Oberkochen microscope fitted with an HBO-100 mercury-arc lamp. Photomicrographs were taken with an Olympus camera containing ASA 400 35mm black and white film.

C activity. Preoperative and postoperative daily serum samples were obtained from each cynomolgus recipient. Total serum C activity (CH50) was determined by incubating serially diluted serum samples with Ab-sensitized sheep red blood cells in Mg++- and Ca++-supplemented buffer after the method of Whaley9. Alternative pathway activity (AP50) was determined by incubating serially diluted serum samples with rabbit red blood cells in buffer containing Mg++ and EGTA. Percent lysis was determined by spectrophotometry.

Anti-porcine Natural Ab levels. Porcine white blood cells (3 x106/ml) were separated from heparinized whole blood on a Ficoll (Organon Teknika Co., Durham, NC) gradient and incubated f r 30 min with 50ml of serially diluted heat-inactivated serum samples. Aft r washing, the cells were incubated with FITC-conjugated goat anti-human IgM and FITC-conjugated goat anti-human IgG (Jackson Immunoresearch,

W st Grove, PA) for 30 min. Th cells were then washed, fixed with 1% formalin, and amount of surface fluorescence analyzed using a FACScan (Becton Dickinson).

Laminin ELISA for anti-Gal Ab levels. Ab directed against the α-galactosyl epitope was assayed by an enzyme linked immunosorbent assay with mouse laminin as a solid-phase antigen as described by Galili et al 10.

Results

Under the conditions described, the control monkey (cyno 1) which received CCS but no systemic sCR1 hyperacutely rejected the Xg in 38 minutes. This is similar to previous results in 5 controls receiving no therapy, all of which rejected within 1 hour . Hyperacute rejection was prevented in cyno 2 (sCR1 + subtherapeutic CCS), but this graft was rejected in an accelerated acute fashion on POD 11. Cynos 3 and 4 (sCR1 + CCS) maintained graft function to POD 21 and 32, respectively, but were sacrificed due to systemic infections. Cyno 3 became septic from gram-positive and gram-negative bacteremia. Cyno 4 developed disseminated CMV (confirmed by histologic examination) with a severe CMV pneumonitis. Both monkeys had functioning grafts at the time of sacrifice.

Complement Levels

All monkeys treated systemically with sCR1 had C activity which was reduced to <10% of preoperative levels as measured by daily CH50 and AP50 assays. C activity remained negligible even when these assays were performed at serum dilutions of 1:2. This is in contrast to the control monkey (cyno 1) which was not given systemic sCR1 but received a graft which was pretreated with sCR1. C activity was modestly suppressed in this monkey after graft reperfusion.

Antibody Levels

All monkeys, except cyno 2, had high levels of circulating preformed Ab prior to transplantation which then dropped precipitously on the day of surgery. A drop in xenoreactive IgM levels occurred in cyno 2, but a decline in IgG levels could not be measured since the preoperative levels of this Ab were negligible.

Becaus cyno 2 received subtherapeutic treatm nt with cyclophosphamide, this monkey never had a WBC < 6,000 cells/mm3. Both IgM and IgG Ab titers began to increase on POD 7 and continued to ris in an accel rated fashion to xceed preoperative levels until POD 11 when the graft ceased contracting.

Cyno 3 had a decline in strength of graft contractions by palpation at 1 week. This was confirmed by noninvasive transabdominal echocardiography to be a regional defect in left ventricular wall motion. Because of the correlation of this event with a rise in Ab levels, the monkey was treated beginning POD11 for rejection with pulsed steroids (Solumedrol 125 mg IV x 2 days followed by a Depomedrol taper from 2mg/kg/day to 1 mg/kg/day IM over 1 week). Graft function stabilized without further decline and Ab titers returned to prerejection levels.

Cyno 4 had low levels of circulating Ab throughout the entire postoperative period and correspondingly excellent graft function.

Ab levels (both IgM and IgG) directed against porcine lymphocytes corresponded with anti-galactosyl Ab levels detected in the laminin ELISA.

Histology

The Xg from cyno 1 had histologic findings of hyperacute rejection with edema, vascular congestion, hemorrhage, and minimal inflammatory infiltrate. Immunofluorescent staining showed intense Ab and moderate C deposition.

The Xg of Cyno 2 had a histologic picture of accelerated acute rejection. This was characterized not only by edema, hemorrhage, and myocardial necrosis but also an inflammatory infiltrate composed primarily of neutrophils (myeloperoxidase+) and macrophages (KP1+) with lesser numbers of T cells (CD3+). Immunofluorescent staining was negative for C, but positive for both IgM and IgG.

Xgs from cynos 3 and 4 had significantly less vascular and myocardial injury than those from the first two monkeys. Additionally, the inflammatory infiltrate was minimal. No significant C deposition was detectable in the grafts, but a vascular staining pattern was apparent for both IgM and IgG.

Rises in xenoreactive Ab levels, particularly IgG, appeared to correlate with rejection episodes. Graft dysfunction occurred in those recipients in which the evoked Ab response closely approached or exceeded preoperative levels. In cyno 2, a rapid rise in Ab on POD7-11 was closely followed by the development of AAR and cessation of graft function. In cyno 3 an early rise in xenoreactiv Ab within the first postoperative week was closely linked to an epis de of AAR and Xg left v ntricular dysfunction. Graft function stabilized following pulsed steroid treatment and a fall in Ab levels.

Cyno 3 had no vidence of graft dysfunction or AAR; Ab lev ls in this monkey remained relatively stable. Although there appeared to be a correlation between Ab levels and AAR episodes, a causal relationship between these two events cannot be definitively established based on these studies. A rise in xenoreactive Ab could be a marker of T cell or NK cell activation or simply an epiphenomenon. Further experiments are necessary to elucidate this relationship.

The presence of Ab and cells within the graft suggests Ab-dependent cellular cytotoxicity. Destruction of Ab coated endothelial cells by Fc receptor positive leukocytes in the absence of C has been demonstrated in an *in vivo* model. Additionally, it has been shown that human NK cells can destroy porcine vascular endothelial cells in the absence of Ab and C13. *In vivo* studies in a C and natural Ab depleted guinea pig-to-rat cardiac xenograft model have suggested that the accelerated rejection that results may be due to the interaction of macrophages and B cells. In this situation, Fc receptor binding of macrophages to xenoreactive Ab produced by sensitized B cells could cause rejection by antibody dependent cell-mediated cytotoxicity.

All donor piglets in this study were administered 25 mg/kg sCR1 IV immediately prior to cardiectomy. The rationale for this came from prior observations of improved ventricular function and more rapid conversion to normal sinus rhythm upon graft reperfusion when donors had received sCR1 pretreatment.

This example confirms that prolonged discordant cardiac xenograft survival can be achieved by C inhibition with sCR1 and triple therapy with standard immunosuppressive agents. It also suggests that if HAR is prevented by C inhibition, and if AAR is prevented for a prolonged period by the addition of immunosuppressive agents, that accommodation can occur in pig-to-primate cardiac xenotransplantation. This example further demonstrates that the addition of routine immunosuppressive agents (cyclophosphamide, cyclosporin, steroids) to C inhibition by sCR1 abates the evoked Ab response, cellular infiltration, and AAR.

Although the inv ntion has been d scribed with reference to the presently preferred embodiment, it should be understood that various m difications can be made with ut departing from the spirit of th invention. Accordingly, the invention is limited only by the following claims.

A number of embodiments of the present invention have been described. Nevertheless, it will be understood that various modifications may be made without departing from the spirit and scope of the invention. Accordingly, it is to be understood that the invention is not to be limited by the specific illustrated embodiment, but only by the scope of the appended claims.

CLAIMS

What is claimed is:

1. A method for transplanting a xenograft to a recipient comprising:

treating a donor with a complement-inhibiting amount of sCR1 prior to xenograft removal;

removing and treating the xenograft to be transplanted with a complement-inhibiting amount of sCR1; and

transplanting the xenograft to the recipient.

2. The method of claim 1, wherein the donor is a porcine animal.

3. The method of claim 1, wherein the recipient is a human.

4. The method of claim 1, further comprising administering to the recipient of the xenograft, substantially contemporaneously with the transplant, an immunosuppressive amount of at least one immunosuppression agent.

5. The method of claim 4, wherein the immunosuppression agent is cyclosporine.

6. The method of claim 4, further comprising administering to the recipient, substantially contemporaneously with the transplant, an antibody-inhibitory amount of cyclophosphamide.

7. The method of claim 4, further comprising administering to the recipient, substantially contemporaneously with the transplant an antibody-inhibitory amount of steroids.

8. The method of claim 1, further comprising administering to the recipient, substantially contemporaneously with the transplant cyclosporine, cyclophosphamide and antibody-inhibitory amounts of steroids.

9. A method of preparing a xenograft comprising treating the xenograft with a complement-inhibiting amount of sCR1 prior to transplantation into a recipi nt.

10. The method of claim 9, wherein the xenograft is *in vivo*.

11. The method of claim 9, wherein the xenograft is in *in vitro*.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/15838

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :A01N 1/02; A61K 39/395, 39/40, 39/42, 39/00, 39/38, 45/00, 45/05
US CL : 424/130.1, 184.1, 278.1; 435/1
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/130.1, 184.1, 278.1; 435/1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	PRUITT, S.K. Effect of Soluble Complement Receptor Type I on Natural Antibody Levels During Xenograft Rejection. Transplantation Proceedings. April 1992, Vol. 24, No. 2, pages 477-478, see entire document.	1-11
Y	PRUITT, S.K. The Effect of Soluble Complement Receptor Type I on Hyperacute Rejection of Procine Xenografts. Transplantation. February 1994, Vol. 57, No. 3, pages 363-370, see entire document.	1-11
Y	ZEHR, K.J. Neutrophil Adhesion and Complement Inhibition Prolongs Survival of Cardiac Xenografts in Discordant Species. Transplantation. March 1994, Vol. 57, No. 6, pages 900-906, see entire document.	1-11

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
24 NOVEMBER 1996	23 DEC 1996
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer HEATHER BAKALYAR, PH.D. Telephone No. (703) 308-0196

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/15838

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	MOORE, F.D. Therapeutic Regulation of the Complement System in Acute Injury States. Advances in Immunology, 1994, Vol. 56, pages 267-299, see entire document.	1-11

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/15838

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

DIALOG: author and word. search terms: xenograft, complement, scr1 or soluble complement receptor?, immunosuppress?, porcine or pig, human, cyclosporine or cyclophosphamide or steroid?
medline, biosis, embase, wpi, scisearch, uspat